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(57) Abstract

A method for identifying a compound inhibiting the phosphorylation activity of a phosphoinositide 3-kinase related kinase polypeptide is described. Antibodies having specific binding affinity for a conjugate including wortmannin are also described.

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SCREENING FOR PHOSPHATIDYLINOSITOL RELATED-KINASE INHIBITORS

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Background of the Invention

The four mammalian members of the phosphatidylinositol kinase-related kinase (PIKK) family function in the regulation of eukaryotic cell-cycle progression and cell-cycle checkpoint functions. Cell-cycle checkpoints ensure that critical events such as DNA replication and chromosome segregation are completed in a timely and accurate fashion during each eukaryotic cell cycle. In addition, certain checkpoints are activated by environmental insults that result in DNA damage, such as ionizing or ultraviolet radiation. Checkpoint activation triggers signal transduction cascades that arrest the progression through the cell cycle to allow repair of the damaged DNA or initiation of programmed cell death. The importance of cell cycle checkpoints in the maintenance of genomic stability is highlighted by the clinical syndrome ataxia-telangiectasia (AT), which results from homozygous loss of function mutations in the ataxia-telangiectasia mutated (ATM) gene. The gene product of the ATM gene, ATM protein, is a member of the PIKK family. AT patients are hyper-sensitive to ionizing radiation and suffer from progressive cerebellar degeneration, immunodeficiency, and a dramatically increased incidence of various cancers, particularly lymphomas. Cells derived from AT patients exhibit defects in G1, S and G2 checkpoints following exposure to

ionizing radiation. These cell cycle checkpoint defects are thought to be causally related to the radiation hypersensitivity and the high mutation rates displayed by AT cells.

The ATaxia and Rad3 related protein (ATR), another member of the PIKK family, shares some checkpoint control functions with ATM, since overexpression of ATR corrects the defective S-phase checkpoint in AT fibroblasts. Cells deficient in ATR kinase activity are also more sensitive to ionizing radiation and exhibit defects in the G2 checkpoint similar to AT cells. Likewise, cells deficient in the catalytic subunit of DNA-dependent protein kinase (DNA-PK_{cs}), another member of the PIKK family, are also hypersensitive to ionizing radiation and exhibit a prolonged G2 arrest following irradiation. DNA-PK_{cs} forms a heterotrimer with Ku70 and Ku80 which is critical for DNA double strand break repair in cells exposed to ionizing radiation and in normal hematopoietic cells undergoing V(D)J gene rearrangements. Unlike the other members of the mammalian PIKK family, rapamycin target protein or mammalian target of rapamycin (mTOR) appears to participate in mitogenic signal transduction. Mammalian TOR protein is also named FRAP or RAFT1. Brown, E.J. et al., Nature 369:756 (1994); Subatini, M. et al., Cell, 78:35 (1994).

Summary of the Invention

The invention is based on the ability of phosphoinositide 3-kinase related kinase (PIKK) polypeptides to phosphorylate PHAS-1 protein. Assays for identifying agents that inhibit the phosphorylation activity of PIKK polypeptides are described. Such inhibitors have therapeutic applications in transplantation, cancer, and other proliferative disorders.

The invention relates to a method for identifying a compound inhibiting the phosphorylation activity of a PIKK polypeptide. The method includes incubating isolated PIKK polypeptide and a substrate of the PIKK polypeptide with the compound to determine if phosphorylation of the substrate is inhibited. The PIKK polypeptide can be, for example, mTOR, ataxia-telangiectasia mutated protein or Ataxia and Rad3 related protein. PHAS-I protein is a particularly useful substrate of the PIKK polypeptides. As used herein, a "compound" refers to a biological macromolecule such as an oligonucleotide or a peptide, a chemical compound, a mixture of chemical compounds, or an extract isolated from bacterial, plant, fungal or animal matter. In particular embodiments, suitable compounds induce radioresistant DNA synthesis in irradiated cells containing the compound.

The invention also features an antibody or fragment thereof having specific binding affinity for a conjugate including wortmannin or an analog thereof and a polypeptide. The antibody can be polyclonal or monoclonal. The polypeptide can be, for example, mTOR, DNA-PK, ataxia-telangiectasia mutated protein or Ataxia and Rad3 related protein.

The invention also relates to a method for identifying a compound that induces radioresistant DNA synthesis within cells. The method includes irradiating the cells, wherein the cells include an effective amount of the compound, and measuring radioresistant DNA synthesis of the cell. The presence or absence of radioresistant DNA synthesis is correlated with activity of the compound.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art

to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting. Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Figure 1 depicts the eIF-4E binding activity of phosphorylated PHAS-I. Radioactivity bound to PHAS-I in each sample lane was quantitated and normalized to the nonphosphorylated control (lane 1). The concentration of wortmannin used in the pretreatment is given in μM.

Figure 2 depicts the phosphorylation of PHAS-1 by immunoprecipitated ATM. Figure 2A depicts ^{32}P incorporation into PHAS-I substrate from A549 lung adenocarcinoma cells and GM 02052 fibroblasts null for ATM expression. Results shown are the mean of two experiments (error bars = s.d.). Figure 2B depicts kinase activity of immunoprecipitated ATM and DNA-PK washed in either kinase buffer (black bar) or high-salt buffer (white bar) toward PHAS-I. Activity was normalized to the kinase buffer washed sample. The mean relative activity from two experiments is shown (error bars = s.d.).

Figures 3A-3C depict the covalent modification and inhibition of ATM, DNA-PK and ATR, respectively, by

wortmannin. Kinase activity was normalized to 1.0 for 0 nM wortmannin treatment. The mean relative activity from two experiments for each kinase is plotted (error bars = s.d.).

Figures 4A-4C depict the inhibition of covalent modification of ATM, DNA-PK and ATR, respectively, by wortmannin in intact cells. Kinase activity was normalized to 1.0 for 0 μM wortmannin treatment. The mean relative activity from two experiments for each kinase is plotted. (error bars = s.d.).

Figure 5 depicts wortmannin-induced radiosensitization of A549 cells as measured by a clonogenic assay. Log-phase cells were exposed to graded doses of radiation and then incubated with DMSO(•), 2 μM(▼), 10 μM (▽) or 20 μM (♦) wortmannin for 14 days prior to fixation and staining. Surviving fraction was calculated relative to the appropriate drug treated, unirradiated control. Results plotted are the mean of four experiments (error bars = SEM).

Figure 6 depicts the effect of wortmannin on radiation-induced G_2-phase delay in A549 cells synchronized in S-phase by treatment with aphidicolin. Histograms of red fluorescence intensity (DNA content) from 20,000 ungated events are shown from a representative experiment. The numbers in each panel indicate the percentage of G_2-M cells in a test population.

Figure 7 depicts the induction of radioresistant DNA synthesis by wortmannin. DMSO (•) or 3 μM (▼), 30 μM (▽) or 100 μM (♦) wortmannin were added to log-phase A549 cells immediately prior to exposure to various doses of irradiation. ^{3}H incorporation was normalized for each treatment to the appropriate drug treated, unirradiated control. Results plotted are the mean of three experiments (error bars = SEM).

Detailed Description

Dysfunction of certain PIKKs, including ATM, DNA-PK, and possibly ATR, leads to defects in DNA damage repair and hypersensitivity to ionizing radiation. As such, PIKK polypeptides are novel molecular targets for the development of agents that, in principle, might sensitize cancer cells to conventional chemotherapeutic agents or ionizing radiation. Indeed, wortmannin is an effective radiosensitizing agent in tumor cells. As described herein, wortmannin-induced radiosensitization is manifested at drug concentrations similar to those required for inhibition of DNA-PK and ATM, but not ATR, in A549 lung adenocarcinoma cells. In addition, the clinically proven immunosuppressive and antiproliferative activities of rapamycin validate mTOR as a target for drug discovery efforts. Identification of PHAS-I as an *in vitro* substrate for the phosphatidylinositol kinase-related kinase (PIKK) polypeptides offers a suitable starting point for the implementation of screens for novel inhibitors of this group of protein kinases.

1.0 Methods for Identifying Compounds Inhibiting PIKK Polypeptides

The invention features a method for identifying a compound that inhibits the phosphorylation activity of a PIKK polypeptide. The method includes incubating isolated PIKK polypeptide and a substrate of the PIKK polypeptide with the compound to determine if phosphorylation of the substrate is inhibited. The PIKK polypeptide can be, for example, mTOR, DNA-PK, ATM or ATR. As used herein, "polypeptide" refers to a chain of amino acids of any length. For example, the PIKK polypeptide can be full-length or can be a single domain of the full-length protein, such as a catalytic or kinase domain. In addition, the PIKK polypeptide can be wild-type or mutant. Mutant PIKK polypeptides are

catalytically active but have decreased binding affinity for regulatory proteins. For example, deletion of amino acid residues 2432 to 2449 of mTOR yields a protein with a 50-100 fold increase in specific activity. Increased specific activity may be desirable for drug screening protocols.

mTOR regulates at least two downstream signaling events in mitogen-stimulated cells. As described herein, one pathway culminates in the phosphorylation of the eIF-4E binding protein PHAS-1, at serine and threonine residues involved in the regulation of eIF-4E binding affinity. The second pathway leads to the phosphorylation and activation of p70S6 kinase (Brown, E.J. et al., Nature 369:756 (1994); Sabatini, D.M. et al., Cell 78:35 (1994); Chen J. et al., Proc. Natl. Acad. Sci. USA 92:4947 (1995)). Both p70S6 kinase and PHAS-I have been implicated in the coupling of growth factor receptor occupancy to increases in protein synthesis. The proposal that mTOR functions as an upstream regulator of the translational machinery receives strong support from genetic studies in budding yeast. Hall, M.N, Biochem. Soc. Trans. 24:234 (1996); Barbet, N.C. et al., Mol. Biol. Cell 7:25 (1996); DiComo, J.C. et al., Genes Dev. 10:1904 (1996). These studies have shown that the rapamycin-sensitive, G_1 progression functions of the yeast TOR proteins are linked to the stimulation of cap-dependent translation.

The rate of progression of mammalian cells through G_1 phase is thought to be governed in part by the ratio of the translational stimulator, eIF-4E, to hypophosphorylated PHAS-I. The functional consequences of an imbalance between these positive and negative regulators of translation are illustrated by the finding that constitutive overexpression of eIF-4E induces malignant transformation of NIH 3T3 fibroblasts.

Lazaris-Karatzas, A. et al., Nature 345:544 (1990); Lazaris-Karatzas, A. et al., Genes Dev. 6:1631 (1992); Rousseau, D. et al., Proc. Natl. Acad. Sci. USA 93:1065 (1996). The transformed phenotype of the eIF-4E-overexpressing cells is partially reversed by genetic manipulations leading to a counterbalancing increase in PHAS-I expression in these cells. Rousseau, D. et al., Oncogene 13:2415 (1996). Thus, hypophosphorylated PHAS-I serves as a negative growth regulator in normal cells, and may function as a tumor suppressor *in vivo*.

Appropriate PIKK polypeptide substrates include PHAS-1 polypeptide and a peptide substrate from the amino-terminus of p53. All mammalian members of the PIKK family can phosphorylate PHAS-I to varying extents. PHAS-1 can be expressed and isolated as described below or can be obtained from Stratagene (La Jolla, CA). Although the actual phosphorylation site(s) in PHAS-I are known only for mTOR, this peptide contains the minimal SQ kinase recognition motif which appears to be shared by ATM and DNA-PK. While phosphopeptide mapping is necessary to confirm that these two proteins share this SQ site as a target for phosphorylation, this overlap in kinase specificity has been described previously for p53 where both ATM[6] and DNA-PK phosphorylate an SQ site at Ser-15 of p53. The minimal recognition motif for ATR remains undefined; however, it does not appear to share the same motif as DNA-PK and ATM since ATR cannot phosphorylate the N-terminal fragment of p53 containing Ser-15. The peptide substrate containing this N-terminal fragment of p53 is 15 amino acids in length and is available from Promega (Madison, WI) or can be chemically synthesized using standard techniques.

1.1 Isolation of PIKK polypeptides

In general, PIKK polypeptides can be isolated using techniques known in the art. For example, the

coding sequence of a PIKK polypeptide can be cloned into a vector and expressed in a host cell. Vectors contain suitable regulatory elements to control the expression of the PIKK polypeptide and are typically a plasmid, cosmid, or a viral vector. Various viral vectors that can be utilized include adenovirus, herpes virus, vaccinia, or, preferably, an RNA virus such as a retrovirus. Preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus, Harvey murine sarcoma virus, murine mammary tumor virus, and Rous Sarcoma Virus. A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated.

Suitable regulatory elements include promoter nucleic acid sequences, enhancer nucleic acid sequences, inducible elements, transcription termination sequences or other control sequences. In general, plasmid vectors contain promoters and control sequences that are derived from species compatible with the host cell. Promoters suitable for use with prokaryotic hosts illustratively include the β-lactamase and lactose promoter systems (Chang, et al., Nature, 275:615, (1978); and Goeddel, et al., Nature, 281:544, (1979), alkaline phosphatase, the tryptophan (trp) promoter system (Goeddel, Nucleic Acids Res., 8:4057 (1980) and hybrid promoters such as the taq promoter (de Boer, et al., Proc. Natl. Acad. Sci. USA, 80:21-25 (1983). However, other functional bacterial promoters are suitable. Their nucleotide sequences are generally known in the art, thereby enabling a skilled worker to ligate them to a polynucleotide encoding the peptide of interest (Siebenlist, et al., Cell, 20:269

(1980) using linkers or adapters to supply any required restriction sites.

Preferred promoters controlling transcription from vectors in mammalian host cells may be obtained from various sources, for example, the genomes of viruses such as polyoma, Simian Virus 40, adenovirus, retroviruses, hepatitis-B virus and most preferably cytomegalovirus, or from heterologous mammalian promoters, e.g. beta actin promoter. The early and later promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment which also contains the SV40 viral origin of replication (Fiers, et al, Nature, 273:113 (1978). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment (Greenaway, et al., Gene, 18:355-360 (1982). Promoters from the host cell or related species also are useful herein.

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman, et al., J. Biol. Chem., 255:2073 (1980) or other glycolytic enzymes (Hess, et al. J. Adv. Enzyme Reg. 7:149 (1968); and Holland, Biochemistry, 17:4900 (1978) such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degraded enzymes associated with nitrogen metabolism, metallothionine, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and

galactose utilization. Yeast enhancers also are advantageously used with yeast promoters.

Enhancer elements include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. Expression vectors that contain a gene which operatively encodes a polypeptide and are intended to be introduced into eukaryotic host cells (yeast, fungi, insect, plant, animal, human or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription which may affect mRNA expression. Examples of suitable selectable markers for mammalian cells which are known in the art include dihydrofolate reductase (DHFR), thymidine kinase or neomycin. When such selectable markers are successfully transferred into a mammalian host cell, the transformed mammalian host cell can survive if placed under selective pressure (i.e., by being conferred with drug resistance or genes altering the nutrient requirements of the host cell).

Construction of suitable vectors containing desired coding, non-coding and control sequences employ standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and relegated in the form desired to construct the plasmids required. Host cells may be transformed with the expression vectors of this invention and cultured in conventional nutrient media modified as is appropriate for inducing promoters, selecting transformants or amplifying genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

For purposes of monitoring expression, recombinant gene expression vectors may be modified to include genes that operatively encode known reporter polypeptides. For example, the pRSV lac-Z DNA vector described in Norton, et al., Mol. Cell. Biol., 5:281 (1985), may produce β-galactosidase with protein expression. Luciferase and chloramphenicol acetyl transferase ("CAT"; see, e.g., Gorman, et al., supra, re construction of a pRSV-CAT plasmid) may also be used. Convenient plasmid propagation may be obtained in *E. coli* (see, e.g., *Molecular Cloning: A Laboratory Manual*)

Expressed polypeptides can be isolated from host cells by conventional chromatographic techniques. For example, gel-filtration, ion-exchange or immunoaffinity chromatography can be used to isolate the proteins. Reverse-phase high performance liquid chromatography (HPLC), ion-exchange HPLC, size-exclusion HPLC, or hydrophobic-interaction chromatography also can be used. See, for example, "Short Protocols in Molecular Biology", Ed. Ausubel, F.M et al., Greene Publishing Associates and John Wiley & Sons, 1992, Chapter 10.

Alternatively, PIKK polypeptides can be isolated by immunoprecipitation. In general, cultured cells are prepared for lysis by washing in phosphate-buffered saline (PBS) prior to harvesting, then incubating with a lysis buffer in the cold and homogenizing, for example, by briefly sonicating. Lysis buffers can include non-ionic detergents such as Nonidet P-40 (NP-40), Igepal CA-630 (Sigma), chelating agents such as EDTA and EGTA, or high or low salt concentrations. Proteolytic activity within a lysate can be minimized by including protease inhibitors such as aprotinin, pepstatin, leupeptin, phenylmethylsulfonyl fluoride (PMSF) and microcystin. A typical lysis buffer can include, for example, 20 mM HEPES buffer, pH 7.4, 1.5 mM $MgCl_2$, 0.15 M NaCl, 1 mM

EGTA, 1 mM dithiothreitol (DTT), and protease inhibitors. After lysis, the lysate is cleared by centrifugation in the cold, i.e. 4°C, at 10,000-12,000 x g for 2-5 minutes, then diluted to an appropriate protein concentration. Antisera can be added to the lysate and incubated on ice for about 1 to about 4 hours. Immune complexes can be precipitated with protein A sepharose beads, then washed in lysis buffer, kinase buffer and a high salt buffer (0.6 M NaCl in 100 mM Tris, pH 7,4) prior to assaying.

1.2 Kinase Assays

Appropriate assay conditions minimally include a buffer and a phosphate donor such as ATP or GTP. For example, a phosphorylation assay can include approximately 10 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 50 mM NaCl, 10 mM MnCl2, 1 mM dithiotheitol (DTT), 10 mM [^{32}P]γ ATP.

Approximately 0.1 μg of PIKK polypeptide and about 1 μg of PHAS-1 can be incubated in the presence of a compound. If a cell extract is the source of PIKK protein, about 100 μg to about 2000 μg of total protein is used. Typically, from about 1 nM to about 1 mM of a compound can be included in the assay. After an initial screening, the optimal concentration of compound can be refined by using an entire range of concentrations.

Kinase assays are typically incubated from about 5 to about 50 minutes in length and are maintained at about 30°C. For example, the incubation can be from about 15 to about 25 minutes. The length of incubation can be adapted to the incubation temperature. For example, if the incubation temperature is about 25°C, incubation time can be increased.

Phosphorylation of PHAS-1 polypeptide can be monitored in various ways, for example by electrophoresing the protein mixture through an SDS polyacrylamide gel, which is then dried and exposed to x-

ray film. The phosphorylation of PHAS-1 polypeptide is compared with a corresponding assay in the absence of compound. Alternatively, inhibition of phosphorylation can be assessed by measuring the amount of radioactivity incorporated into PHAS-1 polypeptide. In this method, the assay is terminated by addition of an equal volume of 30% acetic acid and then spotted onto P-81 phosphocellulose paper (Whatman LabSales, Hillboro, OR) or other suitable material. The paper is then rinsed for five minutes with 1% phosphoric acid and 10 mM sodium pyrophosphate. After four cycles of rinsing, radioactivity is measured by scintillation counting. The phosphocellulose paper can also be washed with 30% trichloroacetic acid (TCA) for 30 minutes at approximately 65°C, then subsequently washed two to four times with 15% TCA for 15 minutes. After drying, the filters are washed in ethanol, dried and counted in a liquid scintillation counter. Casnellie, J.E., Meth. Enzymol., 200:155 (1991).

All the mammalian PIKK family members are inhibited by wortmannin in the low nM to the low μM range. While the mechanism of PIKK inhibition by wortmannin has yet to be defined, it has been extensively studied in PI3K. Wortmannin covalently binds to Lys-802 in the ATP binding domain of PI3K which blocks the binding of ATP in the catalytic cleft. This lysine residue is critical for PI3K kinase activity with a Lys802Arg mutation resulting in a catalytically inactive enzyme. Based on the conservation of this lysine residue throughout the mammalian PIKK family and the irreversible binding of wortmannin to the PIKK members demonstrated in this study, wortmannin may inhibit all PIKK members in a manner similar to PI3K by binding to this crucial lysine residue and thereby acting as a non-competitive inhibitor of ATP binding.

Wortmannin inhibition of DNA-PK has been demonstrated. In contrast to the half-maximal inhibition of DNA-PK at 16 nM as described herein, two other studies have reported IC_{50}s of 250 to 300 nM. These differences in the reported sensitivity of DNA-PK to wortmannin can be explained by the diverse means used for isolating and assaying the DNA-PK catalytic activity. In the study reported below, DNA-PK was immunoprecipitated in a physiologic buffer with mild detergent conditions, which is presumed to preserve the integrity of the DNA-PK heterotrimer. This presumption is supported by the sensitivity of the DNA-PK kinase activity to high salt treatment which has been reported to result in the dissociation of the Ku subunits. Hartley et al. used purified DNA-PK_{cs} in their experiments which is more resistant to inhibition by wortmannin than the intact DNA-PK heterotrimer. Hartley, K.O. et al., Cell, 82:849-856 (1995). In a second study, DNA-PK was not isolated, but assayed directly in cell lysates from SW480 cells treated with wortmannin prior to an *in vitro* kinase assay. The DNA-PK 'specific' peptide substrate used in the kinase assay was derived from the amino-terminus of p53 and includes Ser-15. As described above, ATM can also phosphorylate Ser-15 of p53. Thus, it is possible that the activity measured in these cells lysates was a combination of DNA-PK and ATM kinase activities.

Inhibition of DNA-PK and ATM kinase activity following incubation of intact A549 cells with low micromolar concentrations of wortmannin correlates with the observed dose-response for radiosensitization in the same cell line. Consistent with inhibition of these kinases, wortmannin caused a marked increase in the initial slope and elimination of the shoulder in the radiation survival curve for A549, suggestive of a defect in post-irradiation DNA repair. Such a defect was

reported earlier by Boulton et al. who demonstrated that 20 μM wortmannin produced near-maximal inhibition of DNA double strand break (dsb) repair in Chinese hamster ovary cells following exposure to ionizing radiation. Boulton, S. et al., Carcinogenesis, 17:2285 (1991). This defect in DNA dsb repair led to the speculation that inhibition of DNA-PK was the primary mechanism for wortmannin-mediated radiosensitization.

However, there are now several lines of evidence to suggest that the inhibition of ATM kinase activity contributes to radiosensitization by wortmannin. The demonstration that murine SCID (severe-combined immunodeficiency) cells, which lack functional DNA-PK, are radiosensitized by wortmannin first suggested that additional protein target(s) may be involved in wortmannin-mediated radiosensitization. As shown herein, inhibition of ATM kinase activity is not only seen at radiosensitizing concentrations of wortmannin, but is also associated with multiple cell cycle checkpoint defects. Although the understanding of cell cycle checkpoints is superficial, it is thought that the dysfunction of these checkpoints is responsible for the radiation hypersensitivity observed in AT patients. Similar to AT cell lines, cells treated with radiosensitizing concentrations of wortmannin prior to irradiation undergo a prolonged G2 arrest. Further, the radioresistant DNA synthesis seen following wortmannin treatment is indicative of an abrogation of a S-phase checkpoint and is one of the hallmarks of cells derived from AT patients. More importantly, RDS is not seen in SCID cells which further supports the argument that DNA-PK is not the only target for wortmannin-mediated radiosensitization. Thus, inhibition of both ATM and DNA-PK contribute to the mechanism of radiosensitization by wortmannin.

Wortmannin represents a lead compound in a novel class of radiosensitizers which inhibit signal transduction pathways involved in DNA damage checkpoints and DNA repair. Traditional radiosensitizers achieve synergistic tumor cell killing by either enhancing the level of initial DNA damage caused by radiation or impeding the repair of radiation-induced DNA lesions by inhibiting enzymes involved in DNA metabolism, synthesis and repair. In contrast, the inhibition of checkpoint PIKKs would not only impair the proximal signaling pathways controlling DNA repair, but also would eliminate crucial cell cycle checkpoints that allow time for DNA repair to occur.

2.0 Antibodies

The invention also features an antibody or fragment thereof having specific binding affinity for a conjugate including wortmannin or an analog thereof and a polypeptide. The antibody can be polyclonal or monoclonal. The conjugate can include, for example, wortmannin and a PIKK polypeptide, for example, mTOR, DNA-PK, ATM and ATR.

In general, wortmannin is conjugated to a polypeptide such as ovalbumin and injected into a host mammal. Various host animals can be immunized by injection of a conjugate including wortmannin and a polypeptide. Host animals include rabbits, chickens, mice, guinea pigs and rats. Various adjuvants that can be used to increase the immunological response depend on the host species and include Freund's adjuvant (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin and dinitrophenol. Polyclonal antibodies are heterogenous populations of antibody molecules that are contained in the sera of the immunized animals.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, can be prepared using wortmannin conjugated to a polypeptide such as ovalbumin and standard hybridoma technology.

In particular, monoclonal antibodies can be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture such as described by Kohler, G. et al., Nature, 256:495 (1975), the human B-cell hybridoma technique (Kosbor et al., Immunology Today, 4:72 (1983); Cole et al., Proc. Natl. Acad. Sci USA, 80:2026 (1983), and the EBV-hybridoma technique (Cole et al., "Monoclonal

Antibodies and Cancer Therapy", Alan R. Liss, Inc., pp. 77-96 (1983). Such antibodies can be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the monoclonal antibodies of the invention can be cultivated *in vitro* and *in vivo*.

Antibody fragments that have specific binding affinity for a conjugate including wortmannin or an analog thereof and a polypeptide can be generated by known techniques. For example, such fragments include but are not limited to $F(ab')_2$ fragments that can be produced by pepsin digestion of the antibody molecule, and Fab fragments that can be generated by reducing the disulfide bridges of $F(ab')_2$ fragments. Alternatively, Fab expression libraries can be constructed. See, for example, Huse et al., Science, 246:1275 (1989).

Once produced, the antibodies or fragments thereof are tested for recognition of wortmannin bound to a polypeptide by Western blotting or immunoprecipitation as described herein. Antibodies of the invention are particularly useful for deconvoluting the methods for identifying inhibitors of PIKK proteins. In particular, the antibodies are useful for preventing the re-isolation of wortmannin from the screening assays. Alternatively, the antibodies can be used to identify potential target proteins for wortmannin or analogs thereof in drug-treated cells.

3.0 Radioresistant DNA Synthesis

The invention also features a method for identifying a compound that induces radioresistant DNA synthesis within cells. The method includes irradiating cells, wherein the cells include an effective amount of the compound, and then measuring radioresistant DNA synthesis of the cell. The presence or absence of

radioresistant DNA synthesis is then correlated with the activity of the compound.

Radioresistant DNA synthesis is indicative of an abrogation of an S-phase checkpoint and is measured in the following manner. Cultured cells in exponential growth are harvested and plated in 96-well plates. After approximately 18 hours, cells are irradiated at room temperature with a sufficient dose rate. The irradiated cells are treated as indicated with a compound for approximately 20 minutes at 37°C prior to pulsing with ^{3}H-methyl-thymidine for about 40 minutes. Cells are harvested, transferred onto glass filters and lysed in distilled water. Filter-bound radioactivity is then determined by scintillation counting.

Compounds identified as inducing radioresistant DNA synthesis can be further assessed to determine if they inhibit the phosphorylation activity of a PIKK polypeptide using the above-described methodology.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

Examples

Example 1 - General Methods: Cell Culture, Antisera and Wortmannin Treatment: Human embryonic kidney 293 cells (HEK 293) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2 atmosphere at 37°C. K562 erythroleukemia cells were maintained in RPMI 1640 with 10% FBS. The A549 lung adenocarcinoma cell line was maintained in RPMI 1640 (GibcoBRL) containing 10% FBS. A fibroblast cell line, GM02052, derived from an AT patient, was obtained from the Coriell Institute for Medical Research. GM02052 was maintained in minimal essential medium (GibcoBRL) with 15 mM HEPES, 20% FBS, and 2x non-essential and 2x essential

amino acids (GibcoBRL). Wortmannin (Sigma) was stored at -80°C as a 20 mM stock solution in dimethylsulfoxide (DMSO) and diluted immediately prior to use in either RPMI 1640 for treatment of intact cells or in aqueous buffer. Diluted wortmannin was added directly to the media and incubated at 37°C for one hour prior to lysis for all kinase assays and immunoblotting experiments involving drug treatment of intact cells.

Wortmannin-specific monoclonal antibodies were generated in the Mayo Department of Immunology Monoclonal Antibody Facility by immunizing Balb/c mice with wortmannin conjugated to ovalbumin. The resulting hybridoma supernatants were screened for specificity by an enzyme-linked immunosorbent assay with wortmannin conjugated to bovine serum albumin. On the basis of these initial tests, one antibody-producing hybridoma, designed Wm7.1, was selected for further evaluation. Rat brain extracts were treated with drug vehicle or with 1 μM wortmannin and subjected to SDS-PAGE and immunoblotting with Wm7.1. The results showed that Wm7.1 specifically reacted with a subset of proteins from wortmannin-treated rat brain extracts, but not with control brain extracts. Wm7.1 used in subsequent experiments was purified from ascites fluid by affinity chromatography over Protein G-Sepharose (Pharmacia).

Polyclonal antibodies were generated against the same immunogen in New Zealand White rabbits. Immunizations, boost and antibody bleeds were performed commercially by Cocalico, Inc. Polyclonal antisera were screened for wortmannin-specific immunoreactivity as described above. Rabbit polyclonal antiserum specific for ATR and antibodies specific for ATM (Ab-3) and DNA-PK (Ab-1) were obtained from Oncogene Research-Calbiochem.

Cloning The cDNAs encoding wild type mTOR (mTOR-wt), a rapamycin-resistant mTOR mutant (mTOR-rr), and the

rapamycin-resistant, kinase-dead mTOR double mutant (mTOR-kd) were inserted as restriction fragments into the polycloning region of pcDNA3 (Invitrogen). The Ser^{2035}→Ile substitution that generated the mTOR-rr mutant and the Ser^{2035}→Ile, Asp^{2338}→Ala substitution that generated the mTOR-kd double mutant were created using the Transformer kit (Clontech, Palo Alto, CA). The cDNAs were tagged at their 5'-termini with nucleotide sequences encoding the six amino acid sequence recognized by monoclonal antibody AU1 (Babco, Richmond, CA). The PHAS-I expression vector, pCMV4-PHAS-I is described by Lawrence, J., Adv. Enzyme Regul., 37:239-267 (1997).

Transfections Transfections were performed using TransIT polyamino transfection reagent (Pan Vera Corporation, Madison, WI) according to manufacturer's instructions. Approximately 2 μg pCMV4-PHAS-I and 4 μg of pcDNA3 or pcDNA3 vectors encoding wild-type or mutant mTOR proteins were used to transfect HEK 293 cells seeded into 60 mm dishes at 5 x 10^5 cells per dish. Alternatively, K562 erythroleukemia cells (10^7 cells per sample) were transfected using a BTX model T820 square-wave electroporator. The cells were mixed with 25 μg pcDNA3-mTOR or pcDNA3-mTOR-kd plasmid DNA plus 20 μg pcDNA3 only as filler DNA. Mock transfections were performed with 45 μg pcDNA3 only. The cells were electroporated with a single pulse at field settings of 350 V and 10 msec duration. For p38 MAP kinase assays, K562 cells were transfected with FLAG-p38-encoding plasmid. Control transfections (Co) were performed with pcDNA3 only.

Expression of the transfected mTOR mutants and the phosphorylated forms of PHAS-I was determined by immunoblotting equivalent amounts of cellular protein with AU1 mAb and anti-PHAS-I antibodies, respectively.

Immunoblotting of mTOR and PHAS-1 Transfected cells were cultured for 12 to 16 hours in appropriate medium supplemented with 10% FBS. HEK 293 cells were cultured in DMEM supplemented with 10% FBS for 12 hours, then transferred into low-serum medium containing 0.1% FBS and then cultured for an additional 12 hours, at which time selected samples received either 5 nM rapamycin, the indicated concentrations of wortmannin, or drug vehicle only. The cells were treated for 30 to 60 minutes in culture, and then recombinant human insulin (Gibco BRL, Grand Island, NY) was added to final concentration of 100 nM.

After an additional 30 minutes, cell monolayers were washed with PBS, and harvested by scraping into lysis buffer (50 mM β-glycerophosphate, 1.5 mM ethylene glycol-bis(β-aminoethyl ether)N,N,N',N',-tetraacetic acid (EGTA), pH 7.4, supplemented with 0.5 mM Na_3VO_4, 20 nM microcystin-LR, 0.2mM PMSF, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin, and 1% Nonidet P-40 (NP-40)). Alternatively, cells were osmotically shocked for 10 minutes with 0.4 M sorbitol, then disrupted by sonication in lysis buffer (50 mM Tris HCl, 50 mM β-glycerophosphate, 100 mM NaCl, pH 7.4, 10% glycerol, 1 mM Na_3VO_4, 1 mM DTT, 0.2% Tween-20, and the standard cocktail of phosphatase and protease inhibitors). Post-nuclear detergent extracts were equalized for protein content and were mixed with reducing SDS-PAGE sample buffer.

Duplicate samples were electrophoresed through 12.5% and 7.5% polyacrylamide gels for PHAS-I and AU1 mAb immunoblots, respectively. PHAS-I immunoblots were performed with affinity-purified rabbit polyclonal antibodies generated against a peptide derived from the carboxy-terminus of PHAS-I. Lin, T.A., and Lawrence, J.C., J. Biol. Chem., 271:30199 (1996). AU1-tagged mTOR polypeptides were blotted with AU1 mAb followed by rabbit

anti-mouse IgG antibodies (Pierce). Recombinant p38 was immunoprecipitated with anti-FLAG mAb M1 (Eastman Kodak). The amount of p38 in the anti-FLAG immunoprecipitates was assessed by immunoblotting with a p38-specific antibody (New England Biolabs). Immunoreactive proteins were detected with horseradish peroxidase coupled to protein A followed by chemiluminescence detection using the ECL reagent (Amersham).

Immunoprecipitation and Blotting of ATM, ATR and DNA-PK Late log-phase A549 cells in 100 mm tissue culture dishes were washed twice with PBS and then lysed on ice with scraping in lysis buffer (20 mM Hepes, pH 7.4, 0.15 M NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 10 μg/ml aprotinin, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 20 nM microcystin) containing 0.5% NP-40. Lysates were cleared by centrifugation, pooled if appropriate, and diluted to 2-3 mg/ml. For immunoprecipitations, 2 μl ATR, 8 μg ATM (Ab-3, catalog No. PC#116, Oncogene Research, Calbiochem) and 3 μg DNA-PK antisera were added to 1 ml of lysate and incubated on ice for 1.5 to 2 hours. Fifteen μl packed Protein A-Sepharose beads were added and tubes were rotated for an additional 30 to 60 minutes at 4°C. Immunoprecipitates were washed twice in lysis buffer and twice in kinase base buffer (10 mM Hepes, 50 mM NaCl, 10 mM $MgCl_2$, pH 7.4) prior to incubation with graded doses of wortmannin. Samples were subjected to SDS-PAGE and transferred to Immobilon-PVDF membranes (Millipore) prior to immunoblotting. Wortmannin-bound proteins were detected by probing the membrane with Wm7.1 at 0.8 μg/ml in Tris-buffered saline containing 0.02% Tween-20 (TBST) and 5% milk overnight at 4°C. After washing in TBST, membranes were incubated with a secondary polyclonal rabbit anti-mouse IgG antibody (Pierce). PIKK members were blotted with the appropriate antisera diluted in 5% milk/TBST for 1-2

hours at room temperature. Blots were developed with horseradish peroxidase coupled to protein A and the enhanced Chemiluminescence reagent (Amersham).

Immune Complex Kinase Assays The ATM kinase assay was a modification of a previously described method. A549 cells were lysed for 20 minutes as described above, with the exception that 0.2% Tween-20 was substituted for NP-40 as the detergent and lysates were diluted to 0.5 mg/ml. In experiments involving the AT fibroblasts, cells were harvested by trypsinization and lysates were sonicated to maximize the yield of nuclear proteins. Equivalent amounts of protein (0.5 mg) were incubated on ice for 2 hours with ATM-specific antibodies and precipitated with potential sepharose beads. Following immunoprecipitation, immune complexes were washed twice in lysis buffer with DTT, phosphatase and protease inhibitors, once in a high salt buffer (0.6 M NaCl/0.1 M Tris, pH 7.4) and once in kinase base buffer. When indicated, immunoprecipitates were incubated with graded concentrations of wortmannin for 30 minutes in the dark at room temperature. The kinase reaction mix was then added to give a final concentration of 10 mM Hepes, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, 10 mM [γ-^{32}P]ATP (specific activity: 50 Ci/mmol; ICN) and 25 ng/μl PHAS-I (Stratagene) in a total volume of 40 μl. Kinase reactions were incubated at 30°C for 20 minutes. The phosphorylation of appropriate substrates by each kinase followed linear kinetics under the reaction conditions described. Reactions were terminated with the addition of an equal volume of 30% acetic acid and duplicate aliquots were spotted onto P-81 phosphocellulose paper (Whatman). The papers were rinsed for five minutes with 1% phosphoric acid/10 mM sodium pyrophosphate. After four cycles of rinsing, the radioactivity retained on the paper was measured by scintillation counting.

The DNA-PK kinase assay was similar to the ATM kinase assay with the exception that the lysates were sonicated prior to clearing, and the protein concentration was adjusted to 0.75 mg/ml prior to immunoprecipitation with anti-DNA-PK antibodies. The immune complexes were washed twice with lysis buffer and twice with kinase base buffer prior to the kinase reaction. The kinase reaction conditions were identical to those described above with the exceptions that the reaction time was 15 minutes at 30°C and the substrate was a 15 amino acid DNA-PK peptide substrate derived from p53 (Promega, catalog #V5811). The DNA-PK substrate was used at a concentration of 250 ng/μl per reaction. Samples spotted onto P-81 paper were rinsed with four 5 minute cycles in 15% acetic acid/10 mM sodium pyrophosphate prior to liquid scintillation counting. In experiments evaluating the sensitivity of ATM and DNA-PK kinase activity to high salt, immunoprecipitates were washed twice with lysis buffer and then with either kinase base buffer or a high salt buffer (0.6 M NaCl/0.1 M Tris, pH 7.4). All immunoprecipitates were then washed with kinase base buffer before addition of the kinase reaction mix. In these experiments, PHAS-I was used as the peptide substrate in both the ATM and the DNA-PK kinase reactions.

Cellular extracts for immunoprecipitation of ATR were prepared with the lysis buffer containing 1% Triton X-100. Lysates were diluted to 2 mg/ml protein for immunoprecipitation. The ATR kinase assay was identical to the ATM kinase assay, except the kinase reaction was run for 15 minutes at 30°C and was stopped by the addition of an equal volume of 4x SDS-PAGE loading buffer. Samples were separated by SDS-PAGE, and the proteins were transferred onto Immobilon-PVDF membranes.

The incorporation of ^{32}P into PHAS-I was quantitated with an AMBIS 4000 imaging system.

The protein kinase activity of native mTOR was assayed by immunoprecipitation of this protein from rat brain extracts. Sabers, C.J. et al., J. Biol. Chem., 270:815 (1995). The extracts were supplemented with 1 mM DTT, 0.2 μM microcystin LR, and 10 μg/ml each of leupeptin, pepstatin, and aprotinin. The extracts (1 mg protein per sample) were mixed with affinity-purified rabbit polyclonal antibodies directed against a peptide sequence corresponding to residues 2432-2449 of mTOR. The immune complexes were precipitated with 15 μl protein A-Sepharose beads and the immunoprecipitates were washed two times in immunoprecipitation buffer (50 mM Tris HCl, 50 mM β-glycerophosphate, 100 mM NaCl, pH 7.4, 10% glycerol, 20 nM microcystin LR, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 600 μM PMSF). The precipitates were washed one time in high-salt buffer (100 mM Tris HCl, pH 7.4, 500 mM LiCl), followed by two washes in kinase buffer.

The immunoprecipitates were pretreated with 25 μl of kinase buffer containing 10 μg glutathione S-transferase (GST)-FKBP12 fusion protein and, where indicated, 10 μM rapamycin or 100 μM FK506. Parallel samples were pretreated in a similar fashion with various concentrations of wortmannin. After 40 minutes at room temperature, the beads were washed two times in kinase buffer.

p38 kinase activity toward PHAS-I was assayed as described in Karnitz, L.M. et al., Mol. Cell. Biol., 15:3049 (1995). The amount of p38 in the anti-FLAG immunoprecipitates was assessed by immunoblotting with a p38-specific antibody (New England Biolabs). The immunoprecipitates were treated with GST-FKBP12 plus rapamycin (F•R) or FK506 (F•FK), or with 1 μM wortmannin

(Wm). Immune complex kinase assays were performed with PHAS-I as the substrate. ^{32}P-labelled PHAS-I was detected by autoradiography. Expression of recombinant mTOR proteins was detected by immunoblotting with AU1 monoclonal antibody. The immunoprecipitated FLAG-p38 was detected by immunoblotting with a p38-specific antibody.

Clonogenic Assay The effect of wortmannin on the radiosensitivity of A549 cells was assessed with a clonogenic assay. A549 cells in log-phase were harvested, resuspended in fresh growth medium and plated in triplicate in 60 mm dishes at cell concentrations estimated to result in 20-100 colonies per dish following treatment. Four hours after plating, cells were irradiated at room temperature with ^{137}Cs source at a dose rate of 6.4 Gy/min. Wortmannin was added to the indicated samples immediately after irradiation. The final concentration of the drug solvent did not exceed 0.1% (vol/vol), and this solvent concentration had no effect on either the clonogenicity or radiosensitivity of the A549 cells. Cells were cultured for two weeks prior to fixation and staining with Coomassie Blue. Only colonies with greater than 50 cells were scored. Data shown represent the mean of four independent experiments with error bars representing the standard error of the mean (SEM).

Cell Cycle analysis A549 cells were synchronized at the G_1/S border by culturing for 18 hours in 5 μg/ml aphidicolin (Sigma). Dishes were washed once with PBS and fresh medium was added. At 3.5 hours after release from the aphidicolin block, the S-phase-enriched cells were treated with wortmannin. Thirty minutes later, cells were irradiated with 0 or 5 Gy as described above. After an additional 30 minutes, drug-containing medium was replaced with fresh culture medium. Twenty-four hours later, cells were harvested by trypsinization,

fixed in PBS containing 70% ethanol, and the samples were stored at -20°C. The fixed cells were resuspended in PBS containing 20 μg/ml propidium iodide and 100 μg/ml boiled RNaseA, and were incubated from 30 minutes at 37°C for 30 minutes prior to flow cytometric analysis on a Becton-Dickinson FACScan. Twenty-thousand ungated events were collected. Cell cycle distribution was determined with the ModFit software package (Verity) after excluding doublets and clumps by gating on the DNA pulse height versus pulse area displays.

Radioresistant DNA Synthesis A549 cells in exponential growth were harvested and plated in 96-well plates (10,000 cells per well in 0.1 ml). Each treatment condition was tested in 6 replicate wells. After 18 hours, the cells irradiated at room temperature at a dose rate of 6.4 Gy/min. The irradiated cells were treated as indicated with wortmannin for 20 minutes at 37°C prior to the pulsing with 2 μCi per well ^{3}H-methyl-thymidine (specific activity: 5 Ci/mmol; Amersham) for 40 minutes. Cells were harvested by trypsinization, transferred onto glass filters and lysed in distilled water. Filter-bound radioactivity was determined by scintillation counting. Data shown represent the mean of three independent experiments with error bars representing the standard error of the mean (SEM).

Statistics All statistical analyses were performed using the software program Sigma Plot 4.0 (SPSS). The concentrations resulting in half-maximal inhibition (IC_{50}) for the various kinases were calculated by fitting the data sets with the Hill 4-parameter equation, using a least-squares regression, and then solving the equations for a relative activity of 0.5. The radiation dose-response curves were fit with the linear-quadratic equation: $\ln(S) = -\alpha D - \beta D^2$, where S is the surviving fraction, D is the dose of radiation, and α

and β are constants. Fowler, J.F., Br. J. Cancer, 49:285-300 (1984). The sensitizer enhancement ratio (SER) for each survival curve was calculated as the ratio of radiation doses, that resulted in 10% survival of the cells in the absence or presence of wortmannin. A paired, two-tailed student T-test was used to determine the statistical difference between the calculated SER at each dose of wortmannin as compared to no drug treatment.

Example 2 - Effect of a rapamycin-resistant mTOR mutant on insulin-stimulated PHAS-I phosphorylation: To test whether or not mTOR is an upstream component of the PHAS-I phosphorylation pathway, HEK 293 cells were cotransfected with expression vectors encoding rat PHAS-I and either wild-type (mTOR-wt) or a rapamycin-resistant mTOR (mTOR-rr) mutant that contains a single amino acid substitution ($S^{2035} \rightarrow I^{2035}$) within the FKBP12•rapamycin-binding (FRB) domain. Chen, J. et al., Proc. Natl. Acad. Sci., 92:4947 (1995). This substitution generated a catalytically active version of mTOR that bears a lower binding affinity for the FKBP12•rapamycin complex. To facilitate detection of the wild-type and mutant mTOR cDNA products, both polypeptides were appended at their 5'-termini with a tag sequence recognized by the AU1 monoclonal antibody (mAb). After transfection, the cells were rested in low-serum medium, then treated for 30 minutes with rapamycin prior to stimulation for 30 minutes with insulin. Changes in the phosphorylation state of PHAS-I were monitored indirectly by immunoblotting. Previous studies have shown that phosphorylation of PHAS-I decreases the electrophoretic mobility of this protein during SDS-PAGE. Lin, T.A. et al., Science, 266:653 (1994). Rapamycin treatment inhibited the insulin-stimulated phosphorylation of PHAS-I in mock-transfected or mTOR-wt-transfected 293 cells, as indicated by the decrease in immunoreactivity of the

most highly phosphorylated form of PHAS-I, and by the appearance of hypophosphorylated PHAS-I. In contrast, rapamycin caused no detectable decrease in PHAS-I phosphorylation in mTOR-rr-expressing 293 cells. The ability of the mTOR-rr transfectants to maintain PHAS-I in a hyperphosphorylated state following exposure to the drug suggests that a rapamycin-sensitive activity of mTOR is required for insulin-stimulated PHAS-I phosphorylation in intact cells.

The addition of FKBP12•rapamycin to immune complex kinase assays containing mTOR inhibits the autophosphorylating activity of this kinase *in vitro*. Brown, E. J. et al., Nature, 377:442 (1995). Therefore, a functional mTOR catalytic domain may be needed for the phosphorylation of mTOR in intact cells. As a first approach to test this hypothesis, the impact of wortmannin on PHAS-I phosphorylation in HEK 293 cells cotransfected with either mTOR-wt or the mTOR-rr mutant was assessed. Although wortmannin has been widely used as an inhibitor of PI 3-kinase, this drug also irreversibly inhibits the autophosphorylating activity of mTOR. Brunn, G.J. et al., EMBO J., 15:5256 (1996). The concentration of wortmannin required to inhibit mTOR autokinase activity by 50% (IC_{50}) was approximately 200 nM, and this activity was maximally inhibited by 1 μM wortmannin. Because wortmannin targets the ATP-binding site of mTOR, rather than the FRB domain, the drug can nondiscriminately block the kinase activities of both wild-type mTOR and the rr-mTOR mutant. This prediction was borne out by the finding that 1 μM wortmannin strongly inhibited the phosphorylation of PHAS-I in insulin-stimulated 293 cells transfected with either mTOR-wt or the mTOR-rr mutant. In contrast, pretreatment of the transfected cells with 0.1 μM wortmannin, a drug concentration sufficient to fully inhibit endogenous PI

3-kinase activity (Karnitz, L.M. et al., Mol. Cell Biol., 15:3049 (1995)), failed to suppress the phosphorylation of PHAS-I. The sensitivity of insulin-stimulated PHAS-I phosphorylation to wortmannin is consistent with the idea that a functional mTOR kinase domain is required for this phosphorylation event *in vivo*.

Example 3 - Role of mTOR kinase activity in PHAS-I phosphorylation within intact cells Additional genetic evidence for the involvement of the mTOR kinase domain in PHAS-I phosphorylation was supplied by transfection experiments with a kinase-dead version of the mTOR-rr mutant. The mTOR-rr/kd double mutant contains the rapamycin resistance-conferring $S^{2035}\rightarrow I$ substitution described above, together with a $D^{2338}\rightarrow A$ substitution that inactivates the mTOR kinase domain (Brunn, G.J. et al., EMBO J., 15:5256 (1996)). The 293 cells were cotransfected with PHAS-I and a mTOR-rr/kd expression vector. Control cell populations were cotransfected with pcDNA3 only, or with the pcDNA3 vector encoding the catalytically-active, mTOR-rr mutant. Rapamycin largely eliminates the contribution of endogenous mTOR to the phosphorylation of the transfected PHAS-I protein, and allows a direct comparison of the capacities of the catalytically-active and -inactive mTOR mutants to support this response in intact cells. Whereas PHAS-I was predominantly hyperphosphorylated in mTOR-rr-expressing 293 cells, extensive dephosphorylation of PHAS-I was evident in cells transfected with the catalytically-inactive mTOR-rr/kd double mutant. The decrease in PHAS-I phosphorylation induced by rapamycin treatment of mTOR-rr/kd-expressing cells was similar to that observed in the mock-transfected control cells. These results support the notion that an intact kinase domain is required for the participation of mTOR in the

pathway leading to PHAS-I phosphorylation in intact cells.

Example 4 - Phosphorylation of PHAS-I by immunopurified native mTOR Previous studies have demonstrated that mTOR displays serine-specific autokinase activity in immune complex kinase assays. Brunn, G.J. et al., EMBO J., 15:5256 (1996); Brown, E.J. et al., Nature, 377:442 (1995). To investigate the possibility that PHAS-I itself might serve as a substrate for mTOR, immune complex kinase reactions were performed with mTOR immunoprecipitates from rat brain extracts and recombinant PHAS-I as the substrate. The addition of [γ-^{32}P]ATP to the mTOR-containing kinase reactions resulted in the incorporation of radiolabeled phosphate into PHAS-I, as well as the appearance of more slowly migrating forms of the protein after separation by SDS-PAGE. In contrast, non-immune IgG immunoprecipitates contained no detectable phosphorylating activity toward PHAS-I. The PHAS-I kinase activity present in mTOR immunoprecipitates was strongly inhibited by the addition of FKBP12•rapamycin to the *in vitro* kinase reaction, whereas FKBP12•FK506 had no effect on the phosphorylation of PHAS-I. Furthermore, pretreatment of the immunoprecipitated mTOR with wortmannin suppressed the PHAS-I kinase activity at drug concentrations (0.1 - 1 μM) identical to those required for inhibition of mTOR autophosphorylation *in vitro*. Table 1 presents the amount of [^{32}P]-incorporation into PHAS-I, normalized to that measured in the non-drug treated control.

Table 1

Wortmannin (nM)	[^{32}P] - incorp.
0	100
30	145
100	98
300	56
1000	8

The sensitivity of this phosphorylation reaction to rapamycin and wortmannin strongly supports to the conclusion that mTOR itself is responsible for the phosphorylation of PHAS-I in the immune complex kinase assays.

In subsequent studies, the abilities of recombinant wild-type or kinase-inactive versions of mTOR to phosphorylate PHAS-I *in vitro* were compared. The recombinant proteins were prepared by transfecting K562 erythroleukemia cells with either the mTOR-wt expression plasmid or a plasmid encoding a kinase-dead mutant mTOR (mTOR-kd) bearing the inactivating $D^{2338}\rightarrow A$ substitution in the catalytic domain. After transfection, the AU1-tagged mTOR-wt and mTOR-kd proteins were immunoprecipitated from K562 cell extracts with AU1 mAb. As was observed with the native, rat brain-derived mTOR, recombinant mTOR-wt phosphorylated PHAS-I in immune complex kinase assays. Phosphoamino acid analysis indicated that the phosphorylation occurred on both serine and threonine residues in PHAS-I. In contrast, the level of PHAS-I phosphorylation catalyzed by AU1 immunoprecipitates from mTOR-kd-transfected cells was indistinguishable from the background activity obtained with immunoprecipitates from mock-transfected cells. Moreover, the PHAS-I kinase activity found in mTOR-wt immunoprecipitates was strongly

inhibited by FKBP12•rapamycin or wortmannin, but not by FKBP12•FK506.

In a parallel control experiment, K562 cells were transfected with an expression vector encoding a FLAG epitope-tagged version of the p38 MAP kinase, which, like ERK1 and ERK 2 (Lin, T.A. et al., Science, 266:653 (1994); Haystead, T.A.J. et al., J. Biol. Chem., 269:23185 (1994)), phosphorylates PHAS-I *in vitro*. The FLAG-tagged p38 was immunoprecipitated from cell extracts, and the immunoprecipitates were treated with FKBP12•rapamycin, FKBP12•FK506, or wortmannin prior to the immune complex kinase assay. Although recombinant p38 readily catalyzed the phosphorylation of PHAS-I *in vitro*, p38 kinase activity was not inhibited by FKBP12•rapamycin or 1 μM wortmannin. These results argue that both FKBP12•rapamycin and wortmannin inhibit the PHAS-I kinase activity present in mTOR immunoprecipitates by selectively and directly inhibiting the catalytic activity of mTOR itself.

Example 5 - Phosphorylation of PHAS-I by recombinant, immunopurified mTOR-wt, mTOR-kd protein and FLAG-tagged p38 The effect of mTOR-mediated phosphorylation on the eIF-4E binding activity of PHAS-I was examined by Far Western analysis. Recombinant PHAS-I was incubated with control antibody or anti-mTOR antibody immunoprecipitates from rat brain extracts. Immunoblot analysis of the kinase reaction products demonstrated that exposure to mTOR generated 3 electrophoretically distinct forms of phosphorylated PHAS-I. Pretreatment of the mTOR immunoprecipitates with FKBP12•rapamycin or 1 μM wortmannin blocked both the phosphorylation of PHAS-I and the appearance of PHAS-I forms with retarded electrophoretic mobilities. In contrast, PHAS-I phosphorylation was not inhibited by either rapamycin or FK506 alone, or by the FKBP12•FK506 complex. The same

sample lanes were probed with ^{32}P-labeled eIF-4E in a Far Western blot. Phosphorylation of PHAS-I by mTOR strongly inhibited the eIF-4E-binding activity of PHAS-I in this assay. In accordance with the PHAS-I phosphorylation results described above, the inhibitory effect of mTOR-mediated phosphorylation on PHAS-I binding to eIF-4E was blocked by FKBP12•rapamycin or 1 μM wortmannin (Figure 1). Taken together, these results demonstrate that the phosphorylation of PHAS-I on serine and threonine residues by mTOR downregulates the eIF-4E-binding activity of PHAS-I.

Example 6 - ATM Kinase Assay: Before assessing the sensitivity of the various PIKKs to wortmannin, a series of preliminary experiments were performed to document the specificity of the ATM immune complex kinase assay. A549 cell extracts were immunoprecipitated with preimmune serum or with ATM-specific antibodies as described in Example 1. Samples were then pretreated for 30 minutes at room temperature with 1 μm wortmannin. Immune complex kinase reactions were performed as described in Example 1 and the reaction products were separated by SDS-PAGE. The amount of $^{32}P_i$ incorporation into PHAS-1 substrate was measured with a Molecular Dynamics Phosphorimager System and ImageQuant Software. The results of this experiment are depicted in Table 2. ATM immunoprecipitates from A549 cells phosphorylated PHAS-I to an approximately 25-fold higher level than that catalyzed by control antibody immunoprecipitates.

Table 2

Preimmune Serum	ATM	ATM + Wortmannin
1.0	4.6	0.6

Extracts prepared for an ATM-negative fibroblast line (GM02052) were immunoprecipitated with anti-ATM antibodies. The lack of a detectable kinase activity in ATM immunoprecipitates from the AT fibroblast line GM02052 demonstrated that the kinase activity observed was from ATM or a kinase physically associated with ATM (Figure 2A). To evaluate the possibility of co-precipitation of other PIKKs in the ATM immune complex, α-ATM immunoprecipitates were immunoblotted for DNA-PK or ATR. No DNA-PK or ATR immunoreactivity was detected in the ATM immunoprecipitates. Moreover, the DNA-PK and ATR immunoprecipitates were similarly free of contamination by other PIKK family members. The stability of the ATM kinase activity against PHAS-I, when exposed to a high salt wash, further documented the lack of a contaminating kinase activity in the ATM immune complex. In contrast, DNA-PK phosphorylation of PHAS-I was significantly reduced after a high salt wash, consistent with the dissociation of the DNA-PK heterotrimer of DNA-PK_{cs}, Ku70 and Ku80 (Figure 2B). Thus, based on the absence of ATM kinase activity in an ATM null cell line, the lack of co-association between ATM and either DNA-PK or ATR, and the integrity of the ATM kinase after a high salt wash, it was concluded that the kinase activity seen in ATM immunoprecipitates is due to the intrinsic kinase activity of ATM.

Example 7 - Wortmannin Binds PIKK Protein: To determine the relative potency of wortmannin as an inhibitor of the catalytic activities of DNA-PK, ATM and ATR, appropriate PIKK immunoprecipitates were treated with various concentrations of wortmannin prior to the immune complex kinase assays. As seen in Figure 3A-C, the sensitivities of the PIKKs to wortmannin varied over a wide range, with an IC_{50} of 150 nM for ATM, 16 nM for DNA-PK and 1.8 μM for ATR, respectively. Based on the

covalent interaction of wortmannin with a critical lysine residue in the ATP-binding pocket of PI3K, wortmannin may also inhibit PIKKs by a similar mechanism. To test this hypothesis, a monoclonal antibody (Wm7.1) that specifically recognizes wortmannin bound to protein was developed. Using this antibody, wortmannin binding to DNA-PK, ATM, and ATR was detected by immunoblotting PIKK immunoprecipitates which had been incubated with various concentrations of wortmannin. Moreover, the concentrations of wortmannin at which binding was detected correspond with those necessary for kinase inhibition. These results are highly suggestive that the kinase activities of this PIKKs are indeed inhibited by a covalent interaction with wortmannin.

Example 8 - Inhibition of PIKKs by Wortmannin:

Because of the irreversible inhibition of these kinases by wortmannin, it was possible to evaluate the kinase activity of the PIKKs following incubation of intact cells with wortmannin. This allows a direct correlation between the inhibition of these proteins by wortmannin in intact A549 cells with the dose-response for wortmannin-mediated radiosensitization in these same cells. Following incubation of intact cells with wortmannin, the kinase activities of both ATM and DNA-PK were almost completely inhibited at 30 μM (Figures 4A-B) and were half-maximally inhibited at 5.8 and 3.6 μM, respectively. As expected, minimal binding of wortmannin was detectable by immunoblotting with Wm7.1 following treatment of cells with drug concentrations less than 10 μM. As suggested from the immune complex data, ATR kinase activity was much more resistant to wortmannin in intact cells (Figure 4C), with an IC_{50} of >100μM and the detection of only minimal wortmannin binding on Wm7.1 immunoblots following treatment with wortmannin at concentrations less than 100 μM. As a further test of the specificity of protein

kinase inhibition by wortmannin, A549 cells were treated with wortmannin, and then cyclin-Cdc2 complexes were immunoprecipitated from cellular extracts. The histone H1 kinase activity of Cdc2 was not affected by exposure of A549 cells to 30 mM wortmannin.

Example 9 - Radiosensitization of Cell Lines

Wortmannin has been shown to sensitize a number of human tumor cell lines to radiation. In the A549 cell line used in this study, radiosensitization by wortmannin follows a steep dose-response relationship (Figure 5) with no radiosensitization seen at 2 μM and significant radiosensitization at 20 μM wortmannin. The degree of radiosensitization can be expressed as the ratio of radiation doses that results in a 10% survival, (sensitizer enhancement ratio SER_{10}) which was 2.2 at 20 μM (p=0.02), 1.4 at 10 μM (p=0.05) and 1.0 at 2 μM wortmannin (p=0.43). Treatment with 30 μM wortmannin did not result in a significant additional increase in radiosensitivity as compared to 20 μM. Thus, radiosensitization occurs at concentrations of wortmannin (10-30 μM) which correspond to the inhibition of ATM and DNA-PK, but not ATR kinase activities.

Wortmannin not only increased the sensitivity of A549 cells to radiation, but it also significantly changed the shape of the radiation survival curve. Compared to the DMSO control, 20 μM wortmannin resulted in an eight-fold increase in the initial slope, α, as described by the linear quadratic equation. This increase in α is suggestive of inhibition DNA repair processes that result in the conversion of potentially repairable DNA lesions into non-repairable lesions. Also consistent with inhibition of repair, wortmannin treatment also resulted in a significant flattening of the shoulder of the radiation survival curve which is reflected by an increase in the α/β ratio from 2.0 to

19.5 Gy. The α/β ratio is the dose at which the linear (αD) and quadratic (βD^2) contribution to cell killing are equal and describes how quickly a survival curve begins to bend.

If ATM contributed to the radiosensitizing effect of wortmannin in A549 cells, then the prominent cell cycle checkpoint defects exhibited by AT-deficient cells should be recapitulated in wortmannin-exposed A549 cells. Cells treated with 20 μM wortmannin prior to irradiation showed a marked accumulation of G_2-m-phase cells at 24 hours post-irradiation while non-treated cells displayed a minor increase in the number of G_2-m-phase cells, indicative of a transient G_2 arrest, followed by re-entry into the cell cycle (Figure 6). This prolonged G2-delay is consistent with inhibition of DNA-PK or ATM since cells defective in either kinase exhibit a similar G2 delay. ATM deficient, but not DNA-PK deficient cells, have a defective S-phase checkpoint(s) which results in one of the hallmark characteristics of AT cells: radioresistant DNA synthesis (RDS). As seen in Figure 7, the dose-response relationship for induction of RDS in A549 cells treated with wortmannin correlates with both the inhibition of ATM kinase activity and radiosensitization.

Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

What is claimed is:

1. A method for identifying a compound inhibiting the phosphorylation activity of a phosphoinositide 3-kinase related kinase polypeptide comprising incubating isolated phosphoinositide 3-kinase related kinase polypeptide and a substrate of said polypeptide with said compound to determine if phosphorylation of said substrate is inhibited.

2. The method of claim 1, wherein said polypeptide is mTOR.

3. The method of claim 1, wherein said polypeptide is ataxia-telangiectasia mutated protein.

4. The method of claim 1, wherein said polypeptide is Ataxia and Rad3 related protein.

5. The method of claim 1, wherein said substrate is PHAS-I protein.

6. The method of claim 1, wherein said compound induces radioresistant DNA synthesis in irradiated cells containing said compound.

7. An antibody or fragment thereof having specific binding affinity for a conjugate comprising wortmannin or an analog thereof and a polypeptide.

8. The antibody of claim 7, wherein said polypeptide is mTOR.

9. The antibody of claim 7, wherein said polypeptide is DNA-dependent protein kinase.

10. The antibody of claim 7, wherein said polypeptide is ataxia-telangiectasia mutated protein.

11. The antibody of claim 7, wherein said polypeptide is Ataxia and Rad3 related protein.

12. The antibody of claim 7, wherein said antibody is polyclonal.

13. The antibody of claim 7, wherein said antibody is monoclonal.

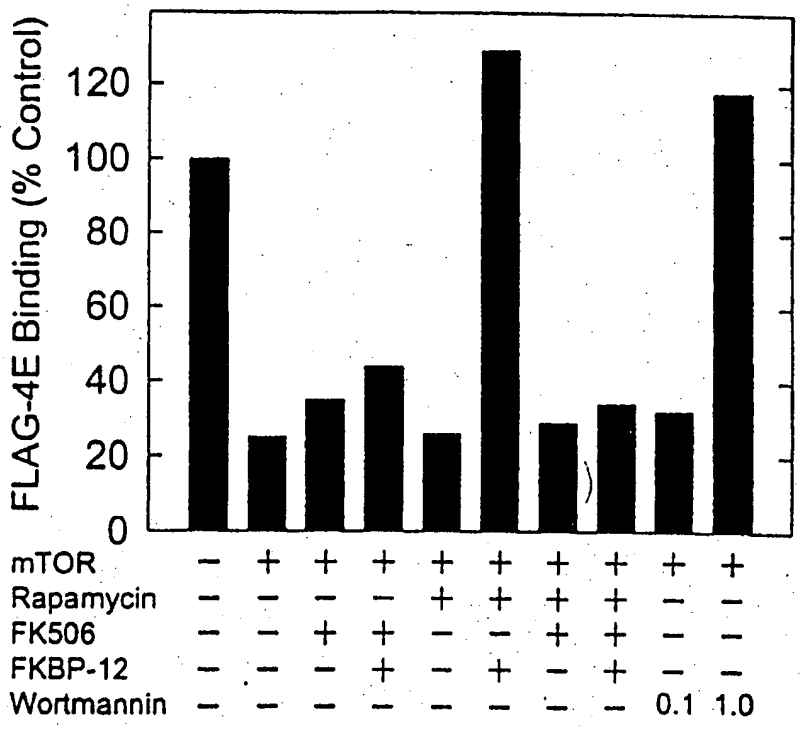
14. A method for identifying a compound that induces radioresistant DNA synthesis within cells, comprising:

(a) irradiating said cells, wherein said cells comprise an effective amount of said compound;

(b) measuring radioresistant DNA synthesis of said cell; and

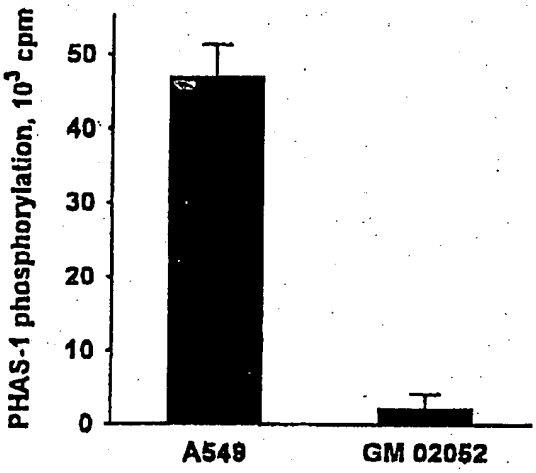
(c) correlating the presence or absence of said radioresistant DNA synthesis with activity of said compound.

FIGURE 1

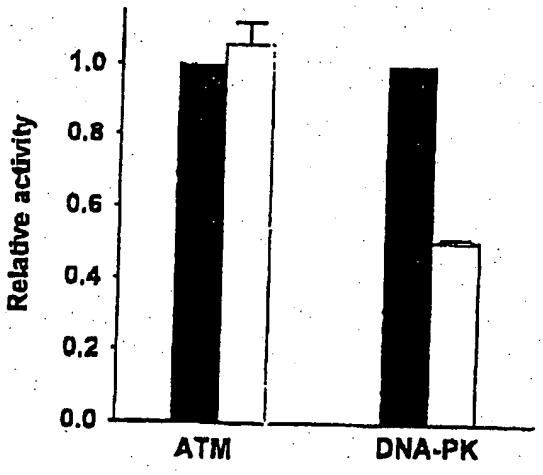

FLAG-4E Binding (% Control)
120
100
80
60
40
20
0
mTOR – + + + + + + + + +
Rapamycin – – – – + + + + – –
FK506 – – + + – – + + – –
FKBP-12 – – – + – + – + – –
Wortmannin – – – – – – – – 0.1 1.0

FIGURES 2A–B

A

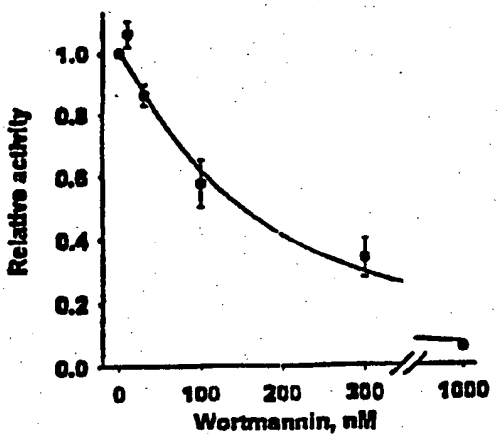


B

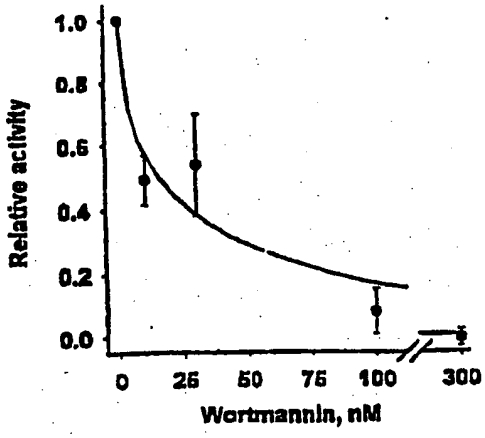


FIGURES 3A-C

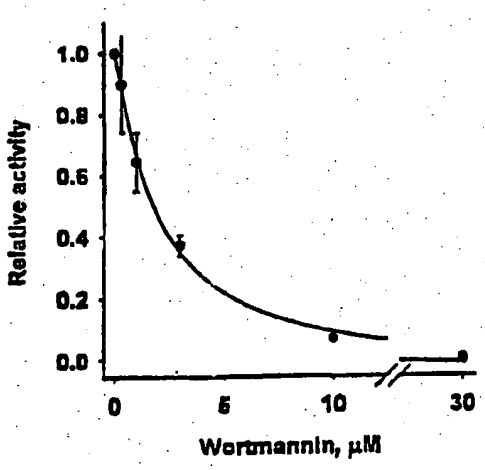
A



B

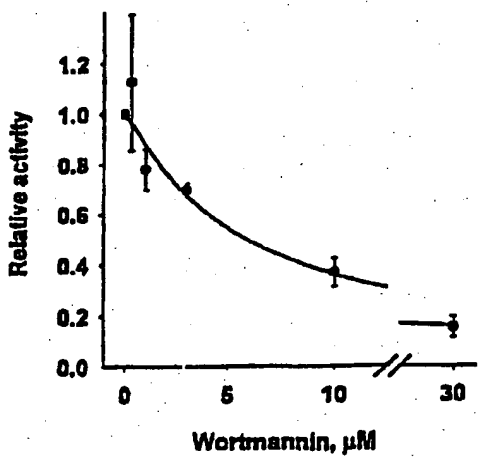


C

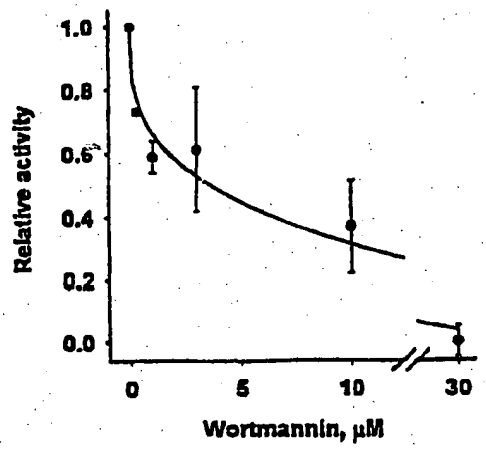


FIGURES 4A-C

A



B



C

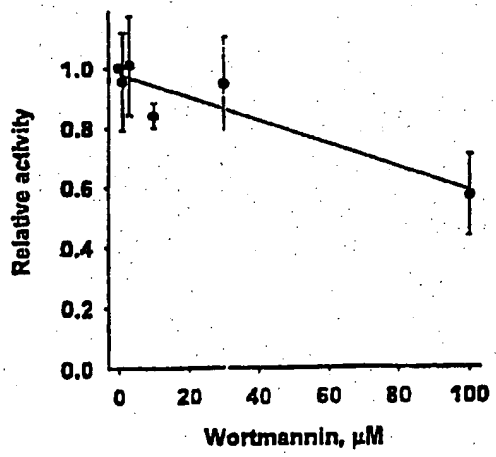


FIGURE 5

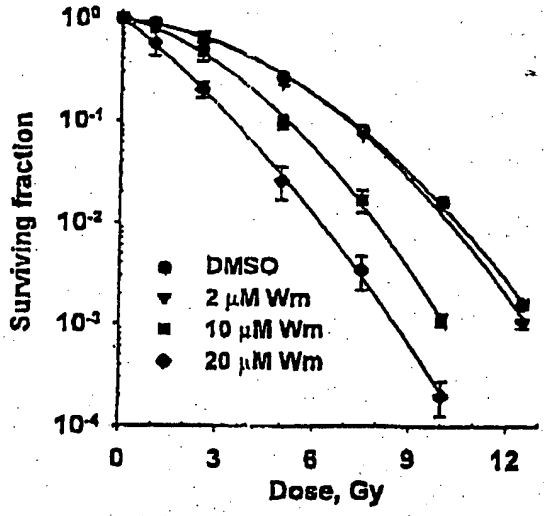

Surviving fraction
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
DMSO
2 μM Wm
10 μM Wm
20 μM Wm
0
3
6
9
12
Dose, Gy

FIGURE 6

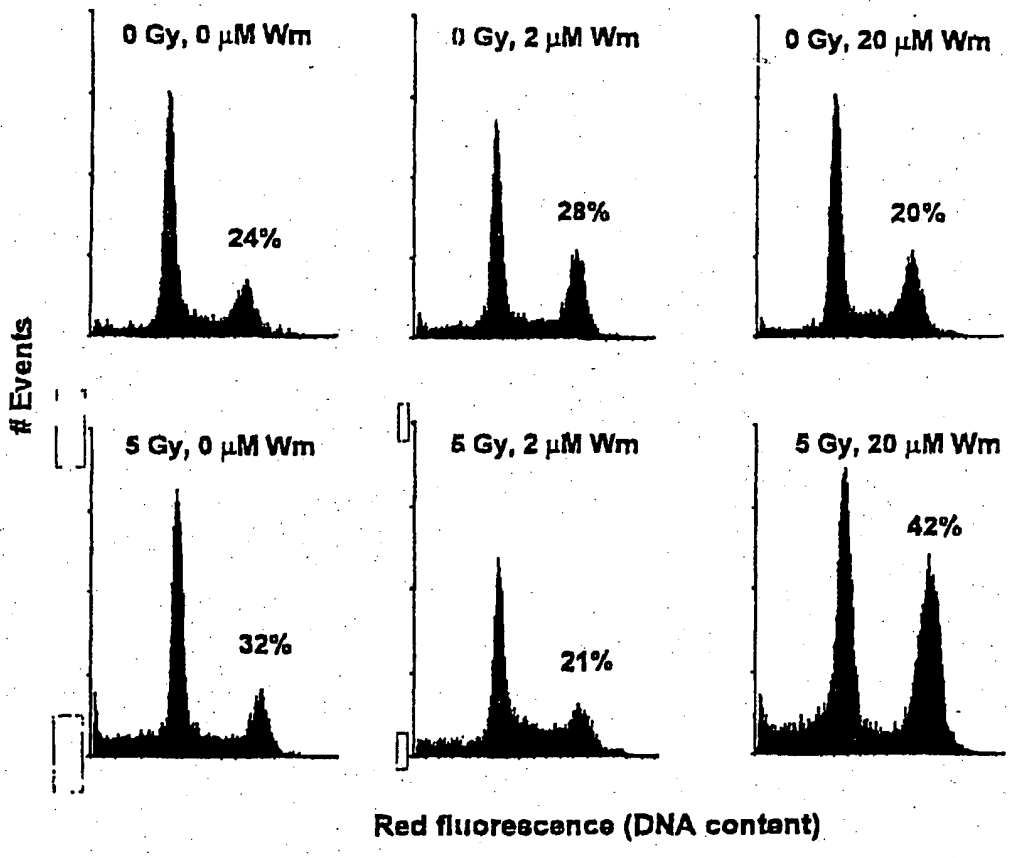
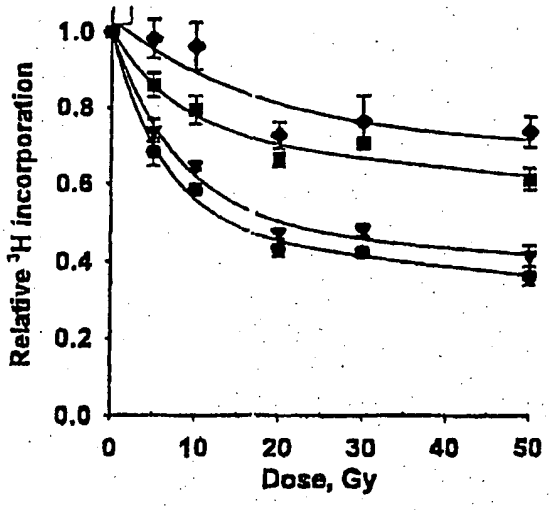

0 Gy, 0 μM Wm
24%
0 Gy, 2 μM Wm
28%
0 Gy, 20 μM Wm
20%
5 Gy, 0 μM Wm
32%
5 Gy, 2 μM Wm
21%
5 Gy, 20 μM Wm
42%
Events
Red fluorescence (DNA content)

FIGURE 7


Relative ³H incorporation
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Dose, Gy

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/11420

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 9/12; C12Q 1/48, 1/50
US CL :435/194, 15, 17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/194, 15, 17

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,466,716 A (IGARASHI et al) 14 November 1995, see entire document.	1-14
A	US 5,151,360 A (HANDA et al) 29 September 1992, see entire document.	1-14

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 03 AUGUST 1998	Date of mailing of the international search report 03 SEP 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer L. BLAINE LANKFORD Telephone No. (703) 308-0196

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